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On the Production of Antibiotics (II) Studies on n-Butylthiomethyl-penicillin*

(Received May 18, 1950)

Tetsuo SUAMI**

Abstract

The recent eminent publication by Behrens et al on the biosynthesis of new penicillins has made the present author to investigate the n-butylthiomethyl-penicillin from the industrial point of view.

The author studies the biosynthetic effects of n-butylmercaptoacetic acid on shaking culture using Penicillium chrysogenum Q 176 pigmentless saltant. The n-butylthiomethyl-penicillin sodium salt is extracted by solvent extraction and purified by chromatography. Using the pure sodium salt of n-butylthiomethyl-penicillin prepared, the stabilities and the effective distribution coefficients between water and organic solvents are estimated.

I. Introduction

The recent eminent publication by Behrens et al[1] on the biosynthesis of new penicillins has made the present author to investigate the n-butylthiomethyl-penicilin from the industrial point of view.

n-Butylmeacaptoacetic acid is prepared by Larsson's method[2] from thioglycolic acid and n-butylbromide and at this juncture the author find that this condensation proceeds more smoothly and rapidly (5 hrs.) in a methanol-water (3:1) solution, yield 62.8%.

The author studies the biosynthetic effects of n-butylmercaptoacetic acid on shaking culture using Penicillium chrysogenum Q 176 pigmentless saltant. The n-butylthiomethyl-penicillin sodium salt is extracted by solvent extraction and purified by chromatography. Using the pure sodium salt of n-buthylthiomethyl-penicillin prepared, the stabilities and the effective distribution coefficients between water and three various organic solvents (butylacetate, ethylether and chloroform) are estimated.

I wish to express may sincere and profound graditude to Prof. Dr. Sumio Umezawa for his continuous and kind guidance. I also extend my heartfelt gratitude to Mr. Masao Naito who kindly helped me in carry out the experiments.

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1) J. Biol. Chem., 175, 771 (1948)

2) Larsson, Ber., 63, 1340 (1930)

II. Experiments

1. n-Butylmercaptoacetic acid

(a) Thioglycolic acid

Three reagents, namely, potassium hydrosulfide[3], sodium hydrosulfide and sodium polysulfide are reacted with monochloroacetic acid[4] respectively. The author find that the first reagent and the second give lower yield (about 45% yield) and the third comperatively better yield. (62.8% yield).

$$2ClCH_2COONa + Na_2S + S \rightarrow \begin{matrix} SCH_2COONa \\ | \\ SCH_2COONa \end{matrix} + 2NaCl$$

$$(SCH_2COOH)_2 + H_2 \rightarrow 2HSCH_2COOH$$

Hot sodium disulfide which is prepared by the addition of 6 gr. of powdered sulfur to 45 gr. of sodium sulfide ($Na_2S \cdot 9H_2O$) fused in a procelain crucible, is added into the mixed solution of 40 gr. of monochloroacetic acid, and saturated water solution of 17 gr. of sodium hydroxide. The temperature of the reaction mixture rises and the reaction finishes in few minutes. The reaction mixture is added slowly with 160 gr. of 50% sulfuric acid and filtered. 30 gr. of zinc tablets are added to the filtrate with agitation and after zinc all disappears, the reaction mixture is filtered to remove zinc sulfate. The filtrate is extracted two times with 50 cc ether, and the ether layer dried over sodium sulfate. The solvent is removed under reduced pressure and residual oil fractionally distilled in vacuo. Thioglycolic acid 27 gr. is obtained, b. p. 123°C/27mm (107—108°C/16mm), yield 70%.

(b) n-Butylbromide

n-Butylbromide is prepared by the method described in Organic Synthesis Vol. I, 28.

(c) n-Butylmercaptoacetic acid

The auther prepares n-butylmercaptoacetic acid by the method of Larsson[3] with 40% yield and tries a modified method, obtaining better yield.

The mixed solution of methanol 300 cc, distilled water 100 cc, sodium hydroxide 20 gr. and thioglycolic acid 25 gr. is added with n-butylbromide 36 gr. under continuous agitation. After 5 hrs. methanol is removed from the reaction mixture by distillation and residual oil is washed with small portions of ether. This is acidified by 20% sulfuric acid and extracted two times with 50 cc ether. The combined ether-extract is dried over sodium sulfate, and ether evaporated under reduced pressure. n-Butylmercaptoacetic acid a colourless 45.2 gr. viscous oil is obtained by distillating the residue in vacuo. b. p. 152°C/20mm yield 62.8%.

2. Submerged fermentation with n-butylmercaptoacetic acid

(a) With 0.018% n-butylmercaptoacetic acid

Penicillium chrysogenum Q 176 pigmentless saltant is inoculated into each flask

3) Klason and Carson, Ber. 39, 732 (1906)

4) Friedlander, Monatsheft f. Chem. 28, 247

of 500 cc volume containing 200 cc of corn steep medium (corn steep 4.0%, lactose 3.0%, glucose 0.5%, calcium carbonate 1.0%, $NaHPO_4$ 0.15%, urea 0.1%, $MgSO_47H_2O$ 0.025%, $ZnSO_47H_2O$ 0.00056%, n-butylmercaptoacetic acid 0.018%, pH 6.5), They are cultivated at 24° ± 1°C on the rotatory shaking machine (150 r. p. m.). On the other hand, the medium without n-butylmercaptoacetic acid is used as the control. The potency is measured by the cylinder-plate method according to F. D. A. penicillin assay method using F. D. A. 209 p as the test organism. The results are shown in Fig. 1.

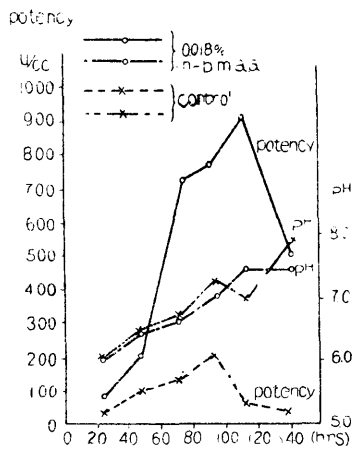

Fig. 1. Potency increase in the medium with 0.018% n-butylmercaptoacetic acid

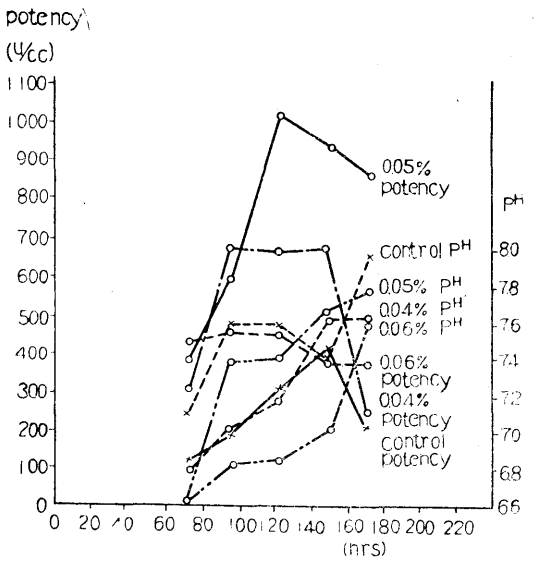

Fig. 2. Influence of the concentration of n-butylmercaptoacetic acid on potency

(b) Optium concentration of n-butylmercaptoacetic acid

To the same medium as (a), 0.04%, 0.05% and 0.06% of n-butylmercaptoacetic acid is added respectively. Penicillium chrysogenum Q 176 pigmentless saltant is used. Fermentation is done in the same conditions as (a). The medium with 0.3% phenylacetic acid instead of n-butylmercaptoacetic acid is used as the control. The results are shown in Fig. 2.

3. Sodium salt of n-butylthiomethyl-penicillin

A 10 L fermentation broth obtained as described in 2 (a) (fermentation 120 hrs., potency 970 u/cc) is cooled with ice, acidified by 10% sulfuric acid and extracted two times with 3.0 L amylacetate at pH 2.0. The amylacetate solution is extract with 2.0 L phosphate-buffer solution (pH 7.0) and then 2.1 L of the first rich-water (potency 4,000 u/cc, total potency 8,400.000 u) is obtained. This first rich-water is extracted with 0.6 L amylacetate two times repeatedly at 0°C, pH

2.0 and total amylacetate solution is titrated with freshly made 5.0% sodium bicarbonate solution to pH 6.8. A second rich water 93 cc (potency 96,000 u/cc, total potency 800,000 u.,) is obtained. The combined yield is 82%.

200 cc of the second rich-water is extracted with 30 cc of amylacetate two times at 0°C, pH 2.0 and combined amylacetate layer is dried over sodium salfate. The dried amylacetate solution is percolated through the colum (diameter 2.1 cm, length 20 cm) of 30 gr. active alumina BL 6 of "Nippon Alminium Co., Ltd." and then the development is done with fresh butylacetate. The column is cutted from the top in eight fractions of 2.5 cm length and each fraction extracted with 50 cc of phosphate-buffer solution (pH 7.0). The results are shown in Table 1.

Table 1

Fraction	Units in Fractions	%	Differential Assay Value*
1	449,000	33.0	0.64
2	429,000	31.5	0.54
3	295,000	21.7	0.53
4	122,000	9.0	—
5	39,000	2.9	—
6	29,300	2.5	—
7	None	0	—
8	None	0	—
Total	1,363,300		

The mixture of the second and the third fraction is extracted with 50 cc butylacetate two times at 0°C, pH 2.0 and total butylacetate is titrated to pH 6.8 with fresh 5% sodium bicarbonate solution.

The final rich-water (110,000 u/cc) 6.2 cc is dried in the frozen state and n-butylthiomethyl-penicillin sodium salt (290 mg, 2.350 u/mg) is obtained.

This is dissolved in 1.5 cc of acetone-water solution (9:1) and after the further addition of 5.0 cc absolute acetone, the solution is cooled in ice. A crystalline sodium salt of n-butylthiomethyl-penicillin salt (200 mg, 2,980 u/mg) is recovered. The potency is elevated by repeated recrystallization (140 mg, 3,310 u/mg). The sodium salt of crystalline n-butylthiomethyl-penicillin represents 0.54 as the differential assay value.

4. Procain salt of n-butylthiomethyl-penicillin

20 cc of the final rich-water described above is added with 1.8 cc of the aqueous solution of 50% procain hydrochloride under agitation and the mixture is stored in refrigerator over night. Prisms are obtained. (potency 1,800 u/mg, yield 803 mg, 84%).

* Test organisms are B. subtilis N. R. R. L. 558 (R) and St. aureous 209 P.

After thrice recrystallization with methanol, n-butylthiomethyl-penicillin procain salt (510 mg) of m. p. 110°C, and potency 2,100 u/mg, is obtained.

5. Calcium salt of n-butylthiomethyl-penicillin

210 mg sodium salt of n-butyltoiomethyl-penicillin (potency 3,310 u/mg, total potency 695,000 u.) is dissolved in 10 cc sterilized distilled water at 0°C and extracted three times with 10 cc butylacetate at pH 2.5. After total butylacetate layer is washed with small portions of chilled water and 200 mg of sedimentary calcium carbonate are added to the butylacetate layer and the mixture are shaken for ten minutes. The aqueous layer which is separated by the centrifugal separator from the mixed solution, is filtered through the Seitz filter and 4.0 cc of the final solntion of n-butylthiomethyl-penicillin calcium salt (potency 130,000 u/cc, total potency 520,000 u, oield 74.8%) is obtained. On drying in the frozen state, 159 mg of calcium salt of n-butylthiomethyl-penicillin (potency 3,230 u/mg, total potency 515,000 u, yield 74%) are obtained. This salt has one molecular water of crystallisation and after drying at 60°C in vacuo, calcium is determined.

Sample : 6,920 mg, drying loss : 0.160 mg, Calcd, for

$C_{14}H_{21}O_4N_2S_2 \cdot \frac{1}{2}Ca \cdot \frac{1}{2}H_2O$: H_2O, 2.41 · Found H_2O, 2.31

Sample : 4.821 mg, 5.423 mg ; $GaSO_4$: 0.904 mg, 1.009 mg ;

Calcd. for $C_{14}H_{21}O_4N_2S_2 \cdot \frac{1}{2}Ca$: Ca 5.49

Found. Ca : 5.49 5.48

6. Stabiliries of n-butylthiomethyl-penicillin in aqueous solution

20 cc of sterilized distilled water in which 13 mg of n-burylthiomethyl-penicillin sodium salt (3,310 u/mg) is dissoved is adjusted to pH 2.0 at 0°C, with 10% sulfuric acid. Maintaining this solution at 0°C, 1.0 cc is taken up from this solution at every interval deluted with phosphate buffer solution (pH 6.5) and immediately assayed by cylinder-plate method. The mean half-value period of n-butylthiomethyl-penicillin is calculated. Results are shown in Table 2 No. 1.

100 cc of sterilized distilled water in which 8.5 mg sodium salt of n-buthylthiomethyl-penicillin (3,310 u/mg) is dissolued is adjusted to pH 2.5, with 10% sulfuric acid at 0°C. The mean half-value period is calculated as shown in Table 2 No. 2.

10 cc of sterilized phosphate-buffer solution (pH 6.5) in which 6.3 mg sodium salt of n-butylthiomethyl-penicillin (3,310 u/mg) are dissoved are maintained at 0°C and assaying this solution at intervals, the mean half-value period shown in Table 2 No. 3 is calculated.

100 cc of sterilized phosphate-buffer solution (pH 6.5) in which 8.5 mg sodium salt of n-butylthiomethyl-penicillin (3,310 u/mg) are dissolved are maintained at 0°C and assaying this solution at intervals, the mean half-value period shown in Table 2 No. 4, is calculated.

Table 2

Hrs	No. 1 pH 2.0		No. 2 pH 2.5		No. 3 pH 6.5		No. 4 pH 6.5	
	u/cc	k×10	u/cc	k×10	u/cc	k×10^2	u/cc	k×10^2
0.0	2,090		270		2,080		281	
1.0	1,800	1.5	–		–		–	
3.0	1,370	1.4	–		–		–	
6.0	–		228	0.28	–		–	
7.0	–		–		2,030	0.44	–	
8.0	660	1.4	–		–		–	
10.0	487	1.4	–		–		–	
12.0	–		193	0.28	1,990	0.43	–	
22.0	–		144	0.29	–		270	0.18
48.0	–		–		–		261	0.15
Mean	$K=1.4\times10^{-1}$		$K=0.28\times10^{-1}$		$K=0.43\times10^{-2}$		$K=0.17\times10^{-2}$	
Half-value period (hrs)	4.9		24.7		157		408	

7. The effective distribution coefficients

5.0 cc of the aqueous solution of vatious concentration of the n-butylthiomethylpenicillin sodium salt (3,310 u/mg) is added with 5.0 cc of organic solvent. The mixture is taken vigorously at 0°C, adjusted to pH 2.5 with 10% phosphoric acid and then aqueous layer is separated by a centrifugal separator. The aqueous layer is diluted with phosphate-buffer solution (pH 6.5) and the effective distribution coefficients are calculated from the potency of the diluted solution. Results are shown in Table 3.

Table 3

Solvents	Potency u/cc			Distribution coefficient
	Initial	Water layer	Organic solvent layer	
Butylacetate	996	16.0	980	61.3/1
〃	204	3.8	200	52.6/1
Ether	996	26.0	970	37.3/1
〃	238	6.1	232	38.0/1
Chloroform	204	10.7	193	18.4/1
〃	238	19.2	219	11.4/1

III. Summary

(1) n-Butylmercaptoacetic acid is prepared by the method of Larsson [2] from thioglychlic acid and n-butylbromide, and at this juncture the author find that this condensation proceeds more smoothly and rapidly (5 hrs.) in a methanol-water (3 : 1) solution, yield 62.8%.

(2) The optimum concentration of n-butylmercaptoacetic acid in culture medium on submerged fermentation using Penicillium chrysogeum Q 176 pigmentless saltant is about 0.05%, and at this condition, a stimulation ratio is 2.46.

(3) The sodium salt of n-butylthiomethy-penicillin (3,310 u/mg) is obtained by chromatographic purification of crude products which is sepatated by solvent extraction from the fermentation broth. The sodium salt is converted into the calcium salt and the analysis of calcium salt gives a satisfactory result.

(4) From the final rich-water, the procain salt of n-butylthiomethyl-penicillin is prepared with procain hydrochloride, and after repeated recrystallization from methanol, prisms obtaind and represent m. p. 110°C and potency 2,100 u/mg.

(5) The stabilities (half-line period) of n-butylthiomethyl-penicillin in aqueous solution at various pH, are determined and it is found that n-butylthiomethyl-penicillin is more stable than penicillin-G.

(6) The effective distribution coefficients of n-butylthiomethyl-penicillin between water and organic solvents at 0°C, pH 2.5 are determined. Butylacetate and ether give larger values and chloroform smaller value than that of penicillin-G.